Differentiation, Distribution, and Elimination of Two Different Pineapple mealybug wilt-associated viruses Found in Pineapple

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ABSTRACT

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Surveys for *Pineapple mealybug wilt-associated virus*-1 (PMWaV-1) and PMWaV-2 were conducted on pineapple samples from Hawaii and around the world. Tissue blot immunoassays (TBIAs) with two different monoclonal antibodies (MAb) specific to either PMWaV-1 or PMWaV-2 indicated that both closteroviruses are widely distributed throughout the pineapple-growing areas of the world. In the worldwide survey, PMWaV-1 was found in 80% of the mealybug wilt of pineapple (MWP)-symptomatic and 78% of the asymptomatic pineapple plants tested. A subset of plants was tested for PMWaV-2; 100% of the symptomatic plants and 12% of the asymptomatic plants were positive for this virus. A reverse transcription-polymerase chain reaction (RT-PCR) assay was developed to differentiate between PMWaV-1 and PMWaV-2. Oligonucleotide primers were designed using distinct regions of the HSP 70 homolog genes of the two viruses. PMWaV-specific RT-PCR assays and TBIAs were used to screen the pineapple accessions maintained at the United States Department of Agriculture-Agricultural Research Service National Clonal Germplasm Repository for PMWaV infection; 73% of the accessions were found infected with at least one PMWaV. Pineapple accessions found PMWaV-free were challenged with viruliferous mealybugs to test for immunity to PMWaV-1. No immune germ plasm was identified. Potential alternative virus hosts were screened for infection with virus-specific RT-PCR assays and TBIAs and were also challenged with viruliferous mealybugs. No alternate hosts of PMWaV-1 or PMWaV-2 were identified. PMWaV-1 infection was eliminated through axillary and apical bud propagation from infected crowns. Strategies to manage MWP are discussed.

Additional keywords: closterovirus, *Dysmicoccus* spp., mealybug wilt of pineapple, MWP, PCV

Mealybug wilt of pineapple (MWP) is currently present in all the major pineapple-growing areas of the world (1,3–5,13,14,25,26). MWP is characterized by severe tip dieback, downward curling, reddening, and wilting of the leaves which can lead to total collapse of the plant (2). Historically, mealybug feeding on the pineapple plant has been associated with MWP symptoms (2,6–8,15,19). In the last two decades, flexuous rod-shaped virus particles, designated *Pineapple mealybug wilt-associated virus* (PMWaV), have been isolated from both symptomatic and asymptomatic pineapple plants (9,12,13,26). PMWaV is actually a complex of at least two different viruses, PMWaV-1 and PMWaV-2 (18) that are transmitted by mealybugs (20,22). Based on particle morphology (9,10) and genomic characteristics (18), it is suggested that PMWaV-1 and PMWaV-2 be placed in the family *Closteroviridae* and in the proposed genus, *Vinivirus* (16,18). PMWaV-1 has previously been detected from both MWP symptomatic and asymptomatic pineapple plants in commercial Hawaiian pineapple fields and in the pineapple germ plasm collection at the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) National Clonal Germplasm Repository (NCGR) in Hilo, HI (12,13). Infection with PMWaV-1 is correlated with growth reductions in the plant crop (20) and yield reductions in the ratoon crop (23). We have recently shown that PMWaV-2 infection and mealybug feeding are necessary for the development of MWP in Hawaii (11,21). The field distribution of PMWaV-2 in Hawaii and worldwide is unknown. In this article, we present survey data for incidence of PMWaV-1 and PMWaV-2 infections in worldwide and local proprietary selections, and introduce reverse transcription-polymerase chain reaction (RT-PCR) assays that detect and distinguish PMWaV-1 and PMWaV-2. We also present survey data for PMWaV-1 and PMWaV-2 incidence in the pineapple germ plasm maintained at the USDA-ARS NCGR and in weed, tree, and shrub species frequently found adjacent to MWP areas in commercial plantings. In addition, we test PMWaV-free germ plasm for PMWaV immunity using viruliferous mealybugs for virus transmission. Last, we demonstrate that PMWaV infection can be eliminated through axillary and apical bud tissue culture propagation from infected crowns.

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MATERIALS AND METHODS

RT-PCR assay and Southern blotting. Pineapple plant leaf tissue (100 mg) or mealybugs (approximately 50) were ground in liquid nitrogen and total RNA was extracted using RNeasy Plant Mini Kits (Qiagen Inc., Chatsworth, CA). Purified RNA samples were stored at –80°C until used. First-strand cDNAs of portions of the PMWaV-1 and PMWaV-2 HSP 70 homolog genes were synthesized from 2.5 to 7 µg of RNA using complementary-strand primers 223 and 226 for PMWaV-2 and PMWaV-1, respectively (Table 1). RT reactions (20 µl) contained 3 µl of RNA template, 0.75 pmols primer, 500 mM dNTPs, 5 mM $MgCl_2$, 4 µl of RT buffer (250 mM Tris-HCl, pH 8.3, 375 mM KC, 15 mM $MgCl_2$, 50 mM DTT), 20 units of RNasin Ribonuclease Inhibitor (Promega, Madison, WI), and 65 units of MMLV reverse transcriptase (Promega). First-strand synthesis was at 42°C (45 min) followed by inactivation at 95°C (5 min). PCRs were carried out in the same tube using primers 224 and 225 for PMWaV-2 and PMWaV-1, respectively (Table 1).

PCRs (50 µl) consisted of 0.75 pmols of sense- and complementary-strand primers, 3 µl of reaction buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0 at 25°C], 1.0% [vol/vol] Triton X-100), and 0.5 µl of *Taq* DNA polymerase (Promega). PCR conditions were initial denaturation at 94°C (4 min), then 45 cycles of 94°C (1 min), 54°C (1 min), and 72°C, (1 min) followed by a final extension at 72°C (10 min) in a Perkin Elmer 480 thermocycler. Amplicons were visualized in 1% agarose gels stained with ethidium bromide. Gels were denatured, neutralized, and transferred in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) overnight onto Zeta-Probe GT membranes (BioRad, Hercules, CA) for Southern blot analysis. α-^{32}PdCTP-labeled probes were synthesized with a Random Primed DNA Labeling System (Life Technologies, Baltimore, MD) using clones 18 and 12 as templates. Clone 18 spanned nucleotides 25 to 983 and clone 12 spanned nucleotides 25 to 948 of the HSP 70 homolog genes of PMWaV-1 and 2, respectively (18). Blots were hybridized overnight at 65°C and washed two times at low stringency (20 mM sodium phosphate, pH 7.2; 5% sodium dodecyl sulfate [SDS]) and two times at high stringency (20 mM sodium phosphate, pH 7.2; 1% SDS) for 30 min each at 65°C. Blots were exposed to Kodak X-OMAT film for 5 to 10 min.

Monoclonal antibody detection of PMWaVs. Nitro ME membranes (Micron Separations, Inc., Westborough, MA) were placed over several layers of paper towels and sandwiched in cardboard cassettes designed to allow blotting of pineapple leaf

Table 1. Primer sequences, amplicon product size, and nucleotide position in the HSP 70 homolog genes of *Pineapple mealybug wilt-associated virus*-1 (PMWaV-1) and PMWaV-2

PMWaV	Primer	Sequence	Product size	Nucleotide[x]
1	225[y]	5′-ACAGGAAGGACAACACTCAC-3′	589	118
1	226[z]	5′-CGCACAAACTTCAAGCAATC-3′	…	707
2	224[y]	5′-CATACGAACTAGACTCATACG-3′	609	226
2	223[z]	5′-TCATTGCACTCACTTATCGTTG-3′	…	835

[x] Primer location (nucleotide position) from beginning of PMWaV HSP70 homolog gene.
[y] Sense-strand primer.
[z] Complementary-strand primer.

Table 2. *Pineapple mealybug wilt-associated virus*-1 (PMWaV-1) and PMWaV-2 infection status of pineapple from around the world

		Symptomatic[v]		Asymptomatic[w]		Unknown[x]	
Source[y]	I.D.[z]	1	2	1	2	1	2
Australia, Queensland	C-10	15/21	NT	0/18	NT	…	…
Australia, Queensland	F-180	10/10	NT	13/13	NT	…	…
Brazil	Perola	1/6	5/5	…	…	…	…
Brazil	Smooth Cayenne	6/15	9/9	…	…	…	…
China, Guangzhou	Cayenne	…	…	…	…	9/54	0/45
Costa Rica	Champaka	9/9	NT	30/55	6/25	…	…
Costa Rica	Cayenne	8/8	NT	7/7	NT	…	…
Costa Rica	C0-2	…	…	1/17	0/16	…	…
Costa Rica	Manzana	…	…	7/7	NT	…	…
Costa Rica	Mayan Gold 3	…	…	3/33	0/30	…	…
Costa Rica	Mayan Gold 4	…	…	3/25	0/22	…	…
Costa Rica	Monte Lirio	…	…	11/12	0/1	…	…
Costa Rica	MacGregor (Queen)	…	…	7/8	0/1	…	…
France, Martinique	Cayenne	…	…	…	…	32/66	7/34
France, Martinique	N.A.	…	…	…	…	10/61	1/51
Guyana, Demerara	Monserrat	52/52	NT	61/61	NT	…	…
Honduras	F-153	20/20	NT	15/19	0/4	…	…
Honduras	Ghanas	…	…	10/10	NT	…	…
Honduras	Ivory Coast	…	…	3/10	7/7	…	…
Honduras	Mayan Gold 1	…	…	59/60	0/1	…	…
Honduras	Mayan Gold 2	…	…	20/28	0/8	…	…
Honduras	Mayan Gold 3	…	…	1/10	0/9	…	…
Honduras	Honduran Field Run	…	…	5/5	NT	…	…
Honduras, La Ceiba	N.A.	60/65	5/5	44/47	0/3	…	…
Honduras	N.A.	101/105	4/4	103/111	2/8	…	…
India	Pundlur	23/23	NT	…	…	…	…
India	Tiptur	31/31	NT	…	…	…	…
Indonesia, Jakarta	Smooth Cayenne	58/62	4/4	53/57	4/4	…	…
Kenya, Thika	Smooth Cayenne	60/64	4/4	18/18	NT	…	…
Malaysia, Pontian, Johor	Gandul	0/95	95/95	…	…	…	…
Malaysia, Pontian, Johor	Mauritius (Queen)	0/5	5/5	…	…	…	…
Philippines, Makati City	Smooth Cayenne	98/101	3/3	98/98	NT	…	…
Philippines, Mindanao	F-200	2/2	…	2/2	…	…	…
Puerto Rico	N.A.	…	50/50	…	…	NT	100/100
South America	N.A.	…	…	…	…	144/144	NT
Sri Lanka, Columbo	Mauritius	…	…	…	…	52/56	4/4
Thailand	Smooth Cayenne	…	…	22/35	0/13	…	…
Vietnam	Queen	…	…	…	…	93/93	NT

[v] Typical mealybug wilt of pineapple (MWP) symptoms included severe tip dieback, reddening, inflexing of margins, and wilting of leaves; NT = not tested. All samples were first screened with PMWaV-1-specific monoclonal antibodies in tissue blot immunoassay because PMWaV-2-specific antibodies were not available at the time. Duplicate blots were not available for screening when PMWaV-2-specific antibodies became available.
[w] No typical symptoms of MWP were present.
[x] MWP symptom status of plants not provided by participant.
[y] Location of plants sampled.
[z] *Ananas comosus* cultivar, variety, or field run selection if known. N.A. = identification not available.

samples onto the membrane while providing rigidity to protect the membrane from breakage. The membranes in the cassettes with illustrated sampling instructions were mailed to pineapple growers and researchers around the world. Participants were asked to make transverse cuts through the basal white portion of pineapple leaves and to press the cut edge onto the membranes. Sample information, such as MWP symptoms, location and age of plant, and variety or cultivar, was also requested. Membranes were returned and processed first with monoclonal antibody (MAb) 35-6-5 for PMWaV-1 (13) and then with MAb 63-2-2 for PMWaV-2 (D. M. Sether and J. S. Hu, *unpublished data*) in tissue blot immunoassay (TBIA) as previously described (12).

Screening of Hawaiian selections and USDA-ARS NCGR accessions. Plants from one *Ananas comosus* clonal selection, five *A. comosus* 'Smooth Cayenne' clonal selections, and four hybrids from Hawaiian plantations were screened for PMWaV-1 and PMWaV-2 infection. Due to the proprietary nature of this material from Del Monte Fresh Produce (Hawaii), Inc., Dole Fresh Fruit Co., and Maui Pineapple Co, clonal selections and hybrids are identified only by numbers, as previously described (12). For each proprietary selection or hybrid, approximately equal numbers of leaf samples were sampled from each field and at least four healthy or MWP-symptomatic fields were sampled for each selection. Asymptomatic plants immediately adjacent in the row to MWP-symptomatic plants were sampled from selection 2. Leaf material was screened in parallel tests for PMWaV-1 and PMWaV-2 with specific MAbs in TBIAs. Infection rates were subjected to Wilcoxon rank sum test. Pineapple accessions maintained at the USDA-ARS NCGR in Hilo, HI, were screened for PMWaV-1 and PMWaV-2 with virus-specific RT-PCR assays and TBIAs as described.

Screening for alternate PMWaV hosts and immune germ plasm. Plants commonly found bordering pineapple fields in Hawaii or that are hosts of mealybugs (*Dysmicoccus brevipes* and *D. neobrevipes*) were established in the greenhouse. Plants (2 to 5 plants of each species) were inoculated with PMWaV-viruliferous mealybugs (*D. brevipes* and *D. neobrevipes*) as previously described (24). Pineapple plants representing PMWaV-free accessions from the USDA-ARS NCGR were also challenged with viruliferous mealybugs. PMWaV-free 'Smooth Cayenne' pineapple plants were included as PMWaV transmission controls. At 4 to 6 weeks after exposure to viruliferous mealybugs, leaves and roots of test plants were assayed by PMWaV-specific RT-PCRs, TBIAs, or both. Two to six plants from woody plant species frequently found growing adjacent to MWP-symptomatic areas of pineapple fields in Hawaii were also sampled. Leaf and petiole material from these plants was tested for PMWaVs with RT-PCR and TBIA.

PMWaV-1 elimination. PMWaV-1-infected pineapple crowns were given two heat treatments consisting of 35°C for 24 h followed immediately by either 58°C for 40 min or 56°C for 60 min in a water bath. Leaves were stripped from the crowns 24 h after the second heat treatment. PMWaV-1-infected crowns that received no heat treatment were included as controls. Axillary and apical buds were excised and surface sterilized in two washes with 15 and 10% bleach and 2 drops of Tween-20 per liter. Axillary buds were trimmed to 1-mm-square blocks while in a 5% bleach solution with Tween-20. These pieces were rinsed in distilled water and placed in media W (Murashige and Skoog Basal Salts [MS salts], with N^6-benzyladenine [BA] at 2 mg/liter, naphthalene acetic acid [NAA] at 2 mg/liter, and agar at 9 g/liter). Buds were transferred twice at 21-day intervals, then placed in B2 media (0.5× MS salts, organic constituents [glycine at 2.0 mg/liter, nicotinic acid at 0.5 mg/liter, pyridoxine HCl at 0.5 mg/liter, thiamine HCl at 0.1 mg/liter], thiamine HCl at 0.4 mg/liter, myo-inositol at 100 mg/liter, BA at 1 mg/liter, and sucrose at 30 g/liter). Once green leaves appeared, plantlets were transferred to Y2 media (0.5× MS salts, organic constituents, thiamine HCl at 0.4 mg/liter, myo-inositol at 100 mg/liter, sucrose at 30 g/liter, and DifcoBacto agar at 9 g/liter) for rooting and then transplanted to soil. Cultures were grown under a daily cycle of 16 h of light and 8 h of dark at 25°C. PMWaV-specific RT-PCR assays were performed prior to transplanting to soil. PMWaV-specific TBIAs were used 1 to 2 months after plants were established in soil.

RESULTS

Worldwide and local surveys. PMWaV-1 and PMWaV-2 were detected in pineapple leaves from around the world with PMWaV-specific TBIAs (Table 2). Both viruses were detected in MWP-symptomatic and asymptomatic plants. All samples from plants described as symptomatic from Costa Rica, Guyana, and India, *A. comosus* F180s from Australia, and *A. comosus* 'Champaka' F153s from Honduras were infected with PMWaV-1. PMWaV-1 was not detected in any symptomatic plants from Malaysia (0 of 100) and was only detected in 33% of the samples from Brazil (Table 2). Specific antibodies for PMWaV-2 were not available during initial screening of the blotted samples from the worldwide survey. When the MAbs for PMWaV-2 became available, the blots that were previously found to be negative with PMWaV-1 antibodies were reprobed with PMWaV-2-specific antibodies in TBIAs. PMWaV-2 was detected in 100% of the samples from MWP-symptomatic plants that were not already found infected with PMWaV-1. PMWaV-2 was also found in 19 of 44 samples (43%) from asymptomatic PMWaV-1-free plants (Table 2).

Surveys of Hawaiian plantations showed a 100% correlation of MWP symptoms with the presence of PMWaV-2 (Table 3) but not with PMWaV-1. Plants growing immediately adjacent to MWP-symptomatic plants in the same row exhibited much higher rates of PMWaV-1 and

Table 3. Detection of *Pineapple mealybug wilt associated virus* (PMWaV)-1 and PMWaV-2 in *Ananas comosus* proprietary selections and hybrids grown in Hawaii

		Percentage of total plants infected[x]		
Description	**Total samples[y]**	**PMWaV-1 only**	**PMWaV-2 only**	**PMWaV-1 and -2**
Asymptomatic				
Hybrid 4	558	10 g	3 hi	3 hi
Hybrid 5	453	10 g	3 hi	5 g
Hybrid 6	666	16 fg	12 fg	53 c
Hybrid 7	40	0 i	0 i	0 i
Selection 1	505	28 de	1 hi	0 i
Selection 2	920	26 e	20 ef	8 g
Selection 3	505	45 c	2 hi	2 hi
Selection 4	505	82 b	1 hi	2 hi
Selection 5	519	99 a	0 i	<1 i
Selection 6	564	34 de	<1 i	36 cd
MWP symptomatic				
Selection 2	385	0 i	0 i	100 a
Selection 5	502	0 i	0 i	100 a
Selection 6	408	0 i	2 hi	99 a
Immediately adj.[z]				
Selection 6	760	0 i	0 i	92 h

[x] PMWaV-1 and PMWaV-2 status was determined by PMWaV-specific tissue blot immunoassays; numbers followed by different letters are significantly different at $P < 0.05$ based on Wilcoxon ranked sum test.

[y] Approximately equal samples were collected from a minimum of four different areas per selection with the exception of Hybrid 4, of which only greenhouse-grown material was sampled.

[z] Plants growing in the same row immediately adjacent to mealybug wilt of pineapple-symptomatic plants.

PMWaV-2 infection than did asymptomatic plants from MWP-free areas of the same field (Table 3). Rates of PMWaV-2 infection in healthy-looking plants of Hawaiian-grown proprietary selections were generally much lower than PMWaV-1 infection rates (Table 3). Comparisons of mean infection rates among crowns collected from seven plant crops and two ratoon crops showed a substantial decrease in PMWaV infection rates in the ratoon crops (Fig. 1). PMWaV-1 and PMWaV-2 were both found in the pineapple accessions maintained at the USDA-ARS NCGR (Table 4). PMWaV-1, PMWaV-2, or mixed infections of both PMWaVs were found in 33, 9, and 40% of the accessions, respectively.

RT-PCR assays. RT-PCR amplicons of 589 bp were produced by primer set 225/226 (Table 1), specific for PMWaV-1 (Fig. 2), when used with RNA extracts from PMWaV-1-infected plants and PMWaV-1 and PMWaV-2 dually infected plants. Amplicons were not present in samples from plants that tested negative for PMWaV-1 with PMWaV-1-specific TBIA or in PMWaV-1-free plants that tested positive for PMWaV-2. Mealybugs reared on squash also tested negative with RT-PCR. RT-PCR amplicons of 609 bp were produced by primer set 224/223 (Table 1), specific for PMWaV-2 (Fig. 2), when used with RNA extracts from PMWaV-2-infected plants, PMWaV-1 and PMWaV-2 dually infected plants, and mealybugs from PMWaV-2-infected plants. Amplicons were not present in samples from plants that tested negative for PMWaV-2 with PMWaV-2-specific TBIA, PMWaV-2-free plants that tested positive for PMWaV-1, or mealybugs reared on squash. Southern blot analyses with ^{32}P-labeled clones 18 and 12, which encompass the 5′-ends of the HSP 70 homolog genes from PMWaV-1 and -2, respectively, showed high homology with the 589-bp (PMWaV-1) or 609-bp (PMWaV-2) amplicons, respectively (Fig. 2). PMWaV-specific RT-PCR could detect virus in the floral bracts of infected plants as well as leaves from the crown or parent plant, whereas PMWaV-specific TBIA could not reliably detect virus in the floral bracts.

Potential alternate hosts and tests for immunity. No PMWaV infections were detected in field-collected samples from herbaceous weeds, shrubs, or trees growing adjacent to MWP-symptomatic pineapple fields (Table 5). Also, no PMWaV infections were detected after exposure to PMWaV-viruliferous mealybugs in agave, banana, cassia, chenopodium, grasses, or tobacco, whereas *A. comosus* Smooth Cayenne controls were infected by PMWaV readily. Several plants commonly found in pineapple fields were found to be hosts of *Dysmicoccus* sp. mealybugs (Table 5). No PMWaV-1-immune germ plasm was identified in the accessions maintained at the USDA-ARS NCGR. All pineapple plants were eventually infected with PMWaV-1, although *A. bracteatus* and *Pseudoananas sagenarius* plants required several applications of viruliferous mealybugs before virus could be detected.

Elimination of PMWaV-1 infection. PMWaV-1 infection was eliminated through apical and axillary bud propagation in 92% of the plants recovered in all treatments (Table 6). Size of crowns showed no differences in percentage of PMWaV-1-free plants recovered. Heat treatments to the crowns prior to bud excision did not improve recovery of PMWaV-1-free plants. Plants that initially tested PMWaV-free with RT-PCR and TBIA assays after virus elimination were grown in soil for over 12 months and remained PMWaV-free based on PMWaV-specific TBIA and RT-PCR assays.

DISCUSSION

PMWaV-1- and PMWaV-2-specific TBIA assays showed that the two closteroviruses are distributed worldwide in both MWP-symptomatic and asymptomatic pineapple. This may indicate inadvertent transportation of systemically infected pineapple propagation material among countries. PMWaV-1 and PMWaV-2 were also found in the pineapple accessions maintained at the USDA-ARS NCGR in Hilo, on the island of Hawaii, and in the Hawaiian proprietary selections and hybrids grown on the islands of Maui and Oahu, where most of Hawaii's pineapple production occurs. In our study with selection number 6, significantly higher PMWaV infection rates were observed in plants immediately adjacent to MWP-symptomatic plants than in plants from healthy-appearing areas within the same field. This indicates that PMWaVs are acquired and transmitted in the field by their mealybug vectors. Mealybugs were always found in areas with MWP.

Only asymptomatic plants of the proprietary hybrids and three of the selections were available for testing at the time of this study. The hybrids are relatively new and have not yet been extensively cultivated; thus, these hybrids have not had the decades of mealybug exposure in the field that the other cultivated selections have had. We are currently testing the hybrids for susceptibility to MWP when exposed to both mealybugs and infected with PMWaV-2. The absence of PMWaV-1 and PMWaV-2 infection in hybrid 7 is likely due to the small number of samples tested and the absence of exposure to viruliferous mealybugs under field conditions. We have been able to infect plants from hybrid 7 with PMWaV-2 using viruliferous mealybugs (D. M. Sether and J. S. Hu, *unpublished data*). MWP has previously been observed in the proprietary selections 1, 3, and 4 (12). In a separate study, MWP-symptomatic plants from these three selections were found to be infected with PMWaV-2 (D. M. Sether and J. S. Hu, *unpublished data*).

RT-PCR assays, confirmed by Southern analyses, were developed to detect and distinguish PMWaV-1 and PMWaV-2 infections. These assays provided a sensitive alternative to immunosorbent electron microscopy for confirming TBIA results. PMWaV-specific RT-PCR assays could detect PMWaV infections in floral bracts, which typically are not good sampling sources for TBIA assays. PMWaV-2 was detected in 100% of the MWP-symptomatic plants in the Hawaiian proprietary selections and in 100% of the MWP-symptomatic plants from around the world that were found to be PMWaV-1 negative. Previously, we found that MWP does not occur without the presence of both PMWaV-2 and mealybug feeding (D. M. Sether and J. S. Hu, *unpublished data*). PMWaV-1 and mealybug feeding does not result in MWP, nor does infection with PMWaV-2 in the absence of mealybug exposure (D. M. Sether and J. S. Hu, *unpublished data*). The correlation of PMWaV-2 infection and MWP in both the Hawaiian and worldwide surveys supports our earlier findings that PMWaV-2 infection is an integral part of MWP etiology.

To date, pineapple of *Ananas* and *Pseudoananus* spp. are the only known plant hosts of PMWaV-1 and PMWaV-2. No alternate PMWaV hosts were identified, although several species in the *Poaceae* and *Liliaceae* families that occur in the borders around pineapple fields were identified as mealybug hosts and were eliminated as sources of PMWaV which could be transmitted to the pineapple crops. The most common weeds were screened for PMWaVs with TBIA and RT-PCR assays and were also challenged with PMWaV-viruliferous mealybugs. We suggest that MWP outbreaks observed on plantations are likely the result of initially non-viruliferous mealybugs, introduced from adjacent alternate mealybug hosts, becoming established on existing PMWaV-2-infected plants. These plants may subsequently develop MWP. Secondary spread of PMWaV then occurs as mealybugs move from infected plants to PMWaV-free plants. Our serological and RT-PCR assays showed that PMWaV infection rates were

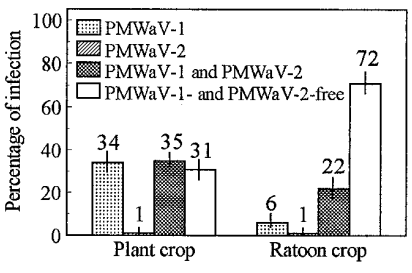

Fig. 1. *Pineapple mealybug wilt-associated virus*-1 (PMWaV-1) and PMWaV-2 infection rates in plant and ratoon crop cycles.

Table 4. *Pineapple mealybug wilt-associated virus* (PMWaV) infection status of pineapple accessions maintained at the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) National Clonal Germplasm Repository in Hilo, HI

			PMWaV-1[o]		PMWaV-2[p]	
No.[q]	Name[r]	Geographic origin	RT-PCR	TBIA	RT-PCR	TBIA
2	59-656	USA	…	+	…	–
3	61-2223	USA	…	+	…	–
4	58-1184	USA	…	+	…	–
6	Tainung #9	Taiwan	…	+	+	+
7	Cayenne lot 520 WBC	USA	…	+	+	+
8	Cayenne Lanai	USA	…	+	–	–
9	Cayenne M263	USA	…	+	+	+
10	Cayenne Hilo	USA	…	+	+	–
11	Columbia variety #1	USA	+	+	-	–
12	'Congo'	USA	…	…	+	+
13	'Spanish Samoa'	American Samoa	+	+	+	+
14	'Pernambuco'	Singapore	+	+	+	+
15	'Ruby'	Singapore	…	+	+	+
16	'Bermuda'	Barbados	…	–	+	–
17	'Natal'	S. Africa	+	+	+	+
18	'Mauritius'	Taiwan	+	+	+	+
20	F101[s]	Brazil	…	+	+	+
21	'Abacaxi'	Brazil	…	+	+	+
23	'Sam Clarke'	Jamaica	+	+	–	…
24	'Monserrat'	Philippines	…	+	…	–
25	'Macgregor'	Philippines	…	–	…	+
27	Wild Kailua, F 148	USA	…	+	…	+
28	'Dacca'	Philippines	+	+	+	+
29	'Sugarloaf'	Philippines	…	+	…	–
30	'Sylhet Jaldubi'	Philippines	–	–	–	–
31	'Black Antigua'	Philippines	…	+	…	+
32	'Cambray'	Philippines	…	+	…	+
33	'Ananas Kendal'	Indonesia	+	+	–	+
34	'Monte Lirio'	Guatemala	…	+	…	+
35	'Amalsad'	India	+	+	–	–
37	'Criolla'	Mexico	…	+	…	+
38	Wild Brazil, F 198[t]	Brazil	–	–	–	–
39	Philippine. Hyb. F 203[u]	Philippines	…	+	…	–
40	'Phu Qui'	Vietnam	…	+	…	+
41	'Pho Lang Tuang'	Vietnam	+	+	+	+
42	'Saigon Red'	Vietnam	…	+	…	+
43	'Mo'	Vietnam	…	–	…	+
44	'Moe'	Vietnam	…	+	…	–
45	'NEP'	Vietnam	–	+	–	–
46	'Sugarloaf'	Zaire	–	+	+	+
47	'Canterra'	Columbia	…	+	…	+
49	'Pina Criolla'	Columbia	–	–	+	+
50	'Bogota'	Columbia	–	–	–	–
51	'Ananas De Vaupes'	Columbia	+	+	+	+
52	'Papuri Vaupes Columbia'	Columbia	…	+	…	–
53	British Samoa P1	Samoa	…	+	…	+
54	British Samoa P5, F243	Samoa	…	+	…	–
55	'Apaporis'	Columbia	…	+	…	–
56	'Apaporis P1'	Columbia	…	+	…	–
58	Wild Brazil × Lot 520[u]	USA	…	–	…	–
59	F1 Cayenne × *P. sagenarius*[u]	USA	–	–	–	–
60	'Spanish Guatemala'	Guatemala	…	+	…	–
62	'Rio Kananari'	Columbia	+	+	–	–
63	CB 2, Curawa[v]	Brazil	–	–	–	–
64	CB 5[s]	Brazil	…	+	…	–
65	CB 6[t]	Brazil	–	–	–	–
66	CB 9[v]	Brazil	+	+	–	–
67	CB 10[t]	Brazil	…	+	…	+

(*continued on next page*)

[o] PMWaV-1 was detected with PMWaV-1-specific primers in reverse transcription-polymerase chain reaction (RT-PCR) and PMWaV-1-specific MAb 35-6-5 in tissue blot immunoassay (TBIA).
[p] PMWaV-2 was detected with PMWaV-2-specific primers in RT-PCR and PMWaV-2-specific MAb 63-2-2 in TBIA.
[q] USDA-ARS National Clonal Germplasm Repository accession number.
[r] Varietal, cultivar, or other identifying name; all are *Ananas comosus* unless footnoted.
[s] *A. bracteatus.*
[t] *A. ananassoides.*
[u] *Ananus* sp.
[v] *A. erectifolius.*
[w] *A. bracteatus* var. rudis.
[x] *A. bracteatus* var. tricolor.
[y] *A. nanus.*
[z] *A. bracteatus* var. albus.

Table 4. (*continued from preceding page*)

No.[q]	Name[r]	Geographic origin	PMWaV-1[o] RT-PCR	PMWaV-1[o] TBIA	PMWaV-2[p] RT-PCR	PMWaV-2[p] TBIA
68	CB 11[s]	Brazil	-	–	–	–
69	CB 15[t]	Paraguay	+	+	+	+
70	CB 17[w]	Paraguay	–	–	–	–
72	CB 19[t]	Paraguay	–	–	–	–
73	CB 20[w]	Paraguay	–	–	–	–
74	CB 21[x]	Paraguay	–	–	–	–
75	CB 23[s]	Argentina	–	–	–	–
76	CB 30	Brazil	…	+	…	+
77	CB 32	Brazil	…	+	…	+
78	CB 36	Brazil	…	+	…	+
79	F1 Hyb. Campines, CB 42[u]	Brazil	…	+	…	–
80	F1 Hyb. *A. ananassoides* × 'Rondon'[u]	USA	…	+	…	+
81	'Rondon'	Brazil	…	+	…	+
83	CB 61[t]	Brazil	–	–	–	–
84	CB 63[y]	Brazil	…	+	…	–
86	'Jandaira'	Brazil	…	+	…	–
87	'Rezende'	Brazil	…	+	…	–
88	CB 71[y]	Brazil	…	–	…	–
89	CB 73[z]	Brazil	…	+	…	–
90	'Prazeres'	Brazil	–	–	–	–
91	Trinidad	Trinidad & Tobago	…	+	…	–
92	Cayenne 573	USA	+	+	+	+
93	Cayenne 666	USA	…	+	…	+
94	Cayenne clone 9	USA	…	+	…	+
95	Cayenne 1069	USA	+	+	–	–
97	Cayenne 7898 4N	USA	…	+	…	–
98	Cayenne 45 #5 4N	USA	+	+	+	+
99	Cayenne 31 4N	USA	…	+	…	–
100	Cayenne 59 4N	USA	–	–	–	+
101	Cayenne M 4W	USA	…	+	…	+
102	Cayenne M 24	USA	…	+	…	–
103	Cayenne M 61 Low bloom	USA	…	+	…	–
104	Cayenne M 63 Plus bloom	USA	…	+	…	+
106	Big eye 'Johnson's Clone'	USA	…	+	…	+
107	Cayenne M 105 big eye	USA	…	+	…	+
108	Cayenne seedy # 24	USA	…	+	…	+
109	Cayenne flowering beauty	USA	+	+	+	+
110	Cayenne M 109-5	USA	+	+	+	+
111	Cayenne M 111 seedy fruit	USA	…	+	…	+
112	Cayenne paper leaf	USA	…	+	…	–
113	Cayenne M 262	USA	+	+	+	+
114	Cayenne bottleneck	USA	…	+	…	+
115	Cayenne M 226 nubby	USA	…	+	…	+
116	Cayenne CPC big eye	USA	…	+	…	–
117	Cayenne M 35	USA	…	+	…	–
119	Cayenne M 267 dry sweet	USA	…	+	…	+
120	'Los Banos'	unknown	–	–	+	+
121	'Amarillo'	Brazil	–	–	–	–
122	'Uhi'	Taiwan	…	+	…	+
123	'Red Spanish'	Panama	…	+	…	+
124	'Taboga'	Panama	+	+	+	+
125	Jamaica Sugar'	Jamaica	…	+	…	–
126	'Smooth Anpi'	Taiwan	–	–	+	+
127	'Kohi'	Taiwan	–	–	–	–
128	'Spiny Anpi'	Taiwan	–	…	+	+
129	'Philippine Green'	Philippines	–	–	+	…
130	'Klajatan'	Indonesia	+	+	–	–
131	'Ananas Merah'	Indonesia	…	+	…	+
132	'Cheese Pine'	Guatemala	…	+	…	–
133	'Kew'	Philippines	…	+	…	–
134	'Kumta'	India	…	+	…	–
135	'Morada'	Venezuela	…	+	…	+
136	'Spanish Criolla Red'	Venezuela	+	+	–	–
138	'Red Spanish Pina Lisa'	Venezuela	…	+	…	–
139	'Cayenne Azores'	Portugal	…	+	…	+
140	'Pakse'	Vietnam	…	+	…	+
141	'Do'	Vietnam	…	+	…	+
142	'Den'	Vietnam	+	+	+	+
143	'Pina De Castilla'	Columbia	–	–	+	+
144	'Manzana'	unknown	…	+	…	+

(*continued on next page*)

Table 4. (*continued from preceding page*)

No.[q]	Name[r]	Geographic origin	PMWaV-1[o]		PMWaV-2[p]	
			RT-PCR	TBIA	RT-PCR	TBIA
145	'Cabezona'	Puerto Rico	…	+	…	–
146	'Antigua'	Guatemala	–	–	–	–
147	Abacaxi Vermelho'	Brazil	…	+	…	+
148	CB 24	Paraguay	–	–	–	–
149	CB 33	Brazil	–	–	–	–
150	CB 38	Brazil	+	+	–	…
151	CB 46	Brazil	…	+	…	+
152	'Rezende'	Brazil	…	+	…	+
154	CB 67	Brazil	…	+	…	+
155	…	USA	–	–	–	–
156	58-696	USA	–	–	–	–
157	63-759	USA	–	–	–	–
158	57-503	USA	…	–	…	–
159	58-1184	USA	…	+	…	–
160	53-116	USA	–	–	–	–
161	58-474	USA	–	–	–	–
162	Cayenne John Teves	USA	…	+	…	–
163	N91-05	Thailand	…	+	…	–
169	32424, N91-17	USA	…	+	…	–
171	…	…	…	+	…	–
…	Singapore	Malaysia	…	+	…	+

significantly higher in areas with mealybugs and MWP, indicating that transmission of PMWaVs occurs in the field. There is no evidence that PMWaV-1 and PMWaV-2 are mechanically transmitted between pineapple plants. Crown materials used for planting new crops are dipped in an insecticide effective against mealybugs prior to being planted. We suggest that eliminating alternate hosts of mealybugs from areas adjacent to pineapple fields and eliminating PMWaV-2-infected pineapple within the field could serve as alternatives for managing MWP.

Identification of PMWaV-infected plants on the massive scale that would be required for converting a plantation over to PMWaV-2-free planting material would be a major financial and technological undertaking. However, small-scale efforts to reduce PMWaV-2-infected plant material in the field could lessen the chances of MWP occurring in the event that mealybugs infestations do occur. One method for reducing PMWaV-2 source plants is to rogue MWP-symptomatic plants and other pineapple plants in the immediate vicinity of symptomatic plants. The distance that constitutes "immediate vicinity" remains to be established and will likely have to account for mealybug population size, duration of exposure, and initial PMWaV-2 infection rate in the crop. Roguing of MWP-symptomatic plants was a common practice in the Hawaiian pineapple industry until the last few decades, when chemical approaches to mealybug control became routine and the costs of labor increased. It has previously been shown that MWP tends to occur in aggregated patterns (14). We suggest that crowns from plants exhibiting mealybug wilt or plants immediately adjacent to MWP-symptomatic plants should not be used for replanting. Under some conditions, MWP-symptomatic plants can recover and new leaves do not exhibit MWP symptoms (7, D. M. Sether and J. S. Hu, *unpublished data*), yet the plants and the propagules remain infected with virus. This recovery state necessitates identifying and marking MWP areas while they are apparent so that propagules collected at a later time will not be used for new plantings.

PMWaV-2 infection is much less common than PMWaV-1 infection in Hawaii. MWP-symptomatic plants may simply fail to produce fruit or propagules. Previously, we detected a reduction in the number of fruit produced in the ratoon cycle from PMWaV-infected plants relative to PMWaV-free plants (23). We have repeatedly observed lower PMWaV-2 infection rates in crown propagules from healthy-appearing ratoon crops than from crowns produced on healthy-appearing plant crops. Selection against PMWaV-2-infected MWP-symptomatic plants may already be occurring. Crown material is usually harvested simultaneously with the fruit. We have previously shown positive correlations between PMWaV infection and precocious, or premature, fruit matu-

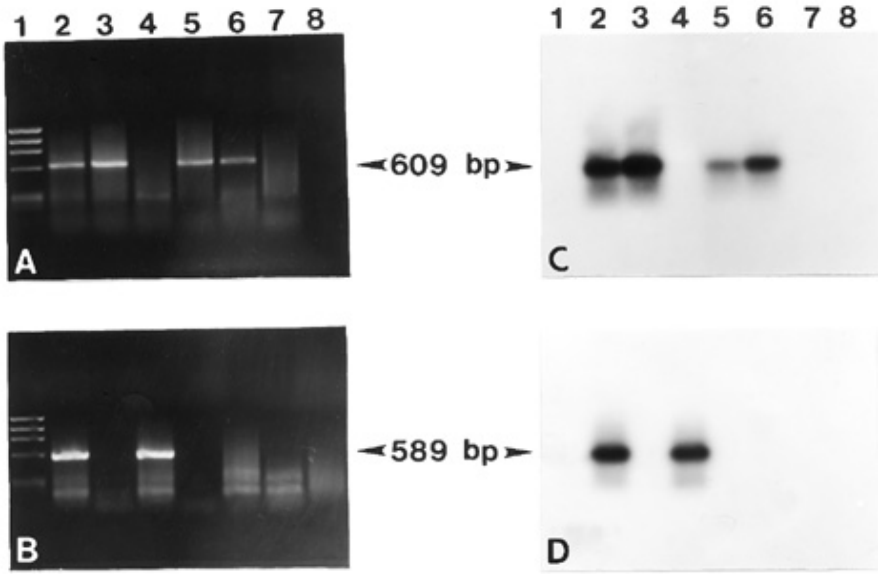

Fig. 2. Detection and differentiation of *Pineapple mealybug wilt-associated virus*-1 (PMWaV-1) and PMWaV-2 with reverse transcription-polymerase chain reaction (RT-PCR) and Southern analyses. RT-PCR analysis with **A,** PMWaV-2- and **B,** PMWaV-1-specific primers. Southern analysis of **C,** PMWaV-2 and **D,** PMWaV-1 ampilcons with clones from the HSP 70 homolog genes of the respective viruses. Lane 1, PhiX 174/Hae III digest marker; lane 2, plant infected with both PMWaV-1 and PMWaV-2; lanes 3 and 5, plants infected with PMWaV-2 only; lane 4, plant infected with PMWaV-1 only; lane 6, mealybugs from PMWaV-2-infected plant; lane 7, PMWaV-1- and PMWaV-2-free plant; lane 8, water control. Plant status was confirmed with PMWaV-specific tissue blot immunoassay.

ration (20). Unless special efforts are made to harvest precocious fruit, these fruit and the accompanying crown can be lost. If MWP plants are fruiting asynchronously with other plants, then crowns from MWP-symptomatic and, concomitantly, PMWaV-2-infected crowns are selected against.

Elimination of PMWaV-1-infection was achieved through apical and lateral bud propagation. Plants derived from infected crowns that were determined to be PMWaV-free following bud propagation remained free of infection after being planted to soil for over 12 months. This method provides a means of recovering PMWaV-free material from infected propagation material. It is especially valuable for pineapple selections which are 100% infected with at least one PMWaV, such as the Hawaiian proprietary selection number 5 (12), and for the accessions maintained at the USDA-ARS NCGR. Treatment of the crowns with heat prior to excising buds did not ensure that buds were PMWaV free. Rather, trimming the bud to a 1-mm square or smaller appeared to be the critical factor for recovering PMWaV-1-free plants. Similar experiments to eliminate PMWaV-2 are in progress.

Table 5. Potential alternate hosts of *Pineapple mealybug wilt-associated virus*-1 (PMWaV-1) and PMWaV-2 challenged with viruliferous mealybugs[w]

Plant	PMWaV-1	PMWaV-2	VMB[x]	Host[y]
Agave sisalana (Engelm.) Perrine (sisal)	0/3	0/3	0/3	+
Albizzia lebbeck (L.) Benth. (siris tree)	0/2	NT	NT	U
Ananas comosus (L.) Merr. 'Smooth Cayenne' (pineapple)	10/10	5/5	3/3	+
Cassia occidentalis L.	0/3[x]	NT	0/3	+/–
Chenopodium quinoa Willd. (lamb's quarters)	0/3[x]	0/3	0/3	–
Chloris radiata (L.) Swartz (finger grass)	0/15	0/10	0/5	+
Citharexylum spinosum (fiddlewood) L.	0/2	0/2	NT	U
Epipremnum pinnatum (L.) Engl.	0/1	NT	NT	U
Eragrostis tenella (L.) Beauv. (Lovegrass)	0/2	0/2	0/1	+
Musa domestica 'Grand Nain' (banana)	0/3	NT	0/3	+
M. domestica 'Williams' (banana)	0/3	NT	0/3	+
Nicotiana benthamiana Domin.	0/3[x]	NT	0/3	+/–
N. clevelandii A. Gray	0/3[x]	NT	0/3	+/–
N. tobaccum (tobacco) L.	0/3[x]	NT	0/3	–
Panicum maximum Jacq. (Guinea grass)	0/6	0/6	0/2	+
Panicum repens L. (Wainaku grass)	0/8	0/8	0/2	+
Paspalum sp.	0/2	0/2	0/2	+
Psidium cattleianum Sabine (strawberry guava)	0/1	NT	NT	U
P. guajava L. (common guava)	0/2	NT	NT	U
Saccharum officinarum L. (sugarcane)	0/5	0/2	NT	+[z]
Sorghum halepense (L.) Pers. (Johnson grass)	0/5	0/5	0/2	+
Trichachne insularis (L.) Nees (Sour grass)	0/3	0/3	NT	U

[w] Plants were tested for PMWaV-1 or -2 with PMWaV-specific reverse transcription-polymerase chain reaction and tissue blot immunoassay; number positive/total number of plants tested; NT = not tested.
[x] Plants were inoculated with PMWaV viruliferous mealybugs (VMB).
[y] + = mealybug host; – = refractory to mealybug feeding; +/– = mealybugs survived on the plant for 5 days but plant was not a preferred host; U = unknown.
[z] Previously identified as a host of *Dysmicoccus brevipes* (17).

Table 6. Elimination of *Pineapple mealybug wilt-associated virus*-1 (PMWaV-1) infection through apical and axillary bud propagation

Selection[u]	Size[v]	1st treatment[w]	2nd treatment[x]	Crowns/buds excised[y]	PMWaV-1-free[z]
5	S	35°C/24 h	58°C/40 min	5/6	13/17 (76%)
5	M	35°C/24 h	58°C/40 min	10/6	15/20 (75%)
2	S	35°C/24 h	56°C/60 min	3/12	8/8 (100%)
2	M	35°C/24 h	56°C/60 min	5/12	9/10 (90%)
2	L	35°C/24 h	56°C/60 min	2/12	13/14 (93%)
5	S	None	None	6/6	14/15 (93%)
5	M	None	None	12/6	33/40 (83%)
5	L	None	None	2/6	9/9 (100%)
2	S	None	None	1/12	6/6 (100%)
2	M	None	None	2/12	9/9 (100%)
2	L	None	None	2/12	9/9 (100%)

[u] Proprietary selection of *Ananas comosus* 'Smooth Cayenne'.
[v] Crowns were graded by size: S = 150 to 200 g, M = 201 to 250 g, and L = 251 to 300 g.
[w] Crowns were submerged in a water bath at a given temperature/duration of treatment.
[x] Immediately following first heat treatment, crowns were submerged at a given temperature/duration of treatment.
[y] Total number of crowns used/total number of excised buds from each crown.
[z] Total number of PMWaV-free plants based on PMWaV-1-specific reverse transcription-polymerase chain reaction assays and tissue blot immunoassays/total number surviving excision; percentage of PMWaV-1-free plants.

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